Observations on Response Factors for Thermal Conductivity Detectors in GLC Analysis of Fatty Acid Methyl Esters

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Abstract

Various correction factors have been proposed for quantitative analysis of fatty acid methyl ester mixtures by isothermal gas-liquid chromatography (GLC) employing thermal conductivity detectors in an effort to obtain more accurate values. These factors include multiplication of the area of each peak by the square root of the molecular weight of each component before calculating the percentage of each compound in the mixture or applying corrections for relative molar response for saturated esters and relative mass response for unsaturated esters. These proposed factors are not valid corrections for detector response but fortuitously are approximate compensations for lack of optimum operating conditions for each component. This is shown from the analysis of a number of standard fatty acid methyl ester mixtures by isothermal and programmed temperature GLC where the detector is maintained under identical conditions. Isothermal GLC requires different factors each time operating conditions are altered appreciably while proper temperature programming required little or no corrections depending on the particular mixture of methyl esters.

Introduction

THE THERMAL CONDUCTIVITY (TC) cell is still the most widely used detector in GLC and the quantitation obtained with this cell has been the subject of many studies. In the analysis of fatty acid methyl esters, composition is often calculated directly from the peak areas and, although the response does not agree with mole or weight per cent, it usually approximates the weight per cent (4,7). In many cases, this accuracy is sufficient; however, for more precise analysis, corrections to the area must be made.

Corrections have taken two forms: (a) empirical calibration techniques and (b) theoretical relationship employing molecular weights. In corrections based on molecular weight or on the number of carbon atoms in the fatty acid chain, it has been stated that the mass response of a TC cell to the methyl esters of saturated fatty acids is a function of molecular weight (10); that is, the response decreases with increasing molecular weight. However, an anomalous behavior is generally recognized with the methyl esters of unsaturated fatty acids on polar columns. On these columns, an increase in unsaturation (a decrease in molecular weight) also results in a decrease in cell response (15).

Thermal conductivity is inversely related to the square root of the molecular weight of a compound (1,9); therefore, as the molecular weight increases, the thermal conductivity decreases. Under GLC conditions, the TC detector is measuring the difference in TC between helium alone (high TC) and the sample in mixture with helium. With each succeeding increase in molecular weight of the sample, there is a greater difference in TC and thus should result in a greater cell response. However, the reverse or lower cell response is generally found in practice. It has been noted in this laboratory and by others (5,18,21) that temperature programming gave area percentage more nearly equal to weight percentage than when isothermal GLC was employed, although the detector, in some instances, was operated under identical conditions for both modes of operation and should have given the same response. This indicated that system conditions were probably responsible for the discrepancy in the analyses and experiments were designed to determine these effects.

Experimental

Materials

The fatty acid methyl esters analyzed were known mixtures obtained from the National Institutes of Health, Bethesda, and Applied Science Laboratories, Inc., State College, while one mixture was prepared in this laboratory from pure methyl esters. These mixtures were prepared by weighing relatively large quantities of each component and the percentage calculated from the weights of each.

Gas-Liquid Chromatography

GLC analyses were performed with a dual column apparatus designed in this laboratory which could be temperature-programmed. It contained a 4-filament thermal conductivity cell (Gow-Mac 9285-Pretzel) which was independently heated in its own compartment and held at a constant temperature of 256C during all analyses. The column temperature for isothermal operation was selected on the basis of good peak separation and reasonable elution time for the most retentive component. For temperature programmed operation, the starting temperature and rate of temperature increase was selected to give good peak separation and as close to equal base widths as possible. Two pairs of columns were employed for analyses. One pair consisted of 2 ft × 3/16 in. (I.D. = 0.124 in.) stainless steel tubing packed with 60–80 mesh Diatoport coated with silicone polymer SE-30 (General Electric), 15% by wt. The second pair was 8 ft × 3/16 in. (I.D. = 0.124 in.) stainless steel tubing packed with 42–60 mesh acid and base washed Chromosorb W coated with 25% by weight ethylene glycol succinate polyester (EGS). Sample size varied from 0.2–0.5 μl.

Since the sensitivity of a thermal conductivity cell is affected by the temperature of the gas flow, it was desirable to bring the temperature of the effluent gas as close as possible to the temperature of the cell before entering the cell proper. For this purpose, 3 ft × 1/16 in. (I.D. = 0.045) stainless steel tubing was inserted between the column exit and the detector. The tubing was tightly wound in a concentric coil with the turns silver soldered to each other; the coil was then sandwiched between the detector and a brass

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TABLE I

Reproducibility of Consecutive Runs
Reference Mixtures—NIH "F"
SE-30 Column—prog. temp. 175–280C (5.6C/min)

Ester	Area %			
	A	B	C	Ave.
14:0	2.6	2.5	2.7	2.6 ± 0.1
16:0	4.4	4.1	4.4	4.3 ± 0.2
18:0	7.6	7.4	7.6	7.5 ± 0.1
20:0	13.5	13.7	13.7	13.6 ± 0.1
22:0	25.5	25.7	25.4	25.5 ± 0.2
24.0	46.5	46.7	46.1	46.4 ± 0.3

block which separated the heater from the TC cell.

An Infotronics CRS-1 digital integrator with automatic baseline corrector, connected directly to the TC detector, was employed to measure peak areas.

Results and Discussion

The level of reproducibility obtained by temperature programming is demonstrated by the data in Table I. The values are three consecutive determinations of the peak area percentages with the deviation from the average. All other results given in this paper are the average of triplicate determinations and their precision is of the order shown.

The results of anaylses of mix "F," available from the National Institutes of Health (NIH), by isothermal operation as compared with temperature-programmed operation are given in Table II. The deviations from the known weight percentage for the isothermal data vary from +0.8 to −1.9 and percentage deviation from +16.0 to −4.1 while with temperature-programming the deviation is only +0.2 to −0.2 and percentage deviation from +4.0 to −4.0 which represents a significant improvement. Since detector temperature and gas flow were identical in both cases, the improvement must have been caused by operation under more favorable column conditions.

Two proposed correction calculations to compensate for "detector response" were applied to the data obtained from mix "F." The first (correction A) was proposed by Horrocks, Cornwell and Brown (15) where the following equation for relative molar response in the saturated methyl ester series was obtained experimentally.

$$R = (24.68 + 5.79\,N - 0.075\,N^2)\ 1.019$$

where R = relative molar response compared with methyl palmitate, N = number of carbon atoms in the fatty acid. (Essentially the same correction was obtained from theoretical considerations using the relationship

$$\frac{R_1}{R_2} = \left(\frac{M_1}{M_2}\right)^{2/3}$$

where M = molecular weight (12).)

The second correction (correction B), proposed by Eastman (6), states that the area of a particular component multiplied by the square root of its molecular weight, divided by the sum of all the areas treated the same way, gives the true weight fraction of the component.

$$\left(\frac{m_i}{W}\right) = \frac{A_i\sqrt{M_i}}{\sum_i A_i\sqrt{M_i}}$$

$$\text{where}\left(\frac{m_i}{W}\right) = \text{weight fraction}$$

of the i^{th} component; A_i = area of the i^{th} component; M_i = molecular weight of the i^{th} component. This equation was applied originally to the GLC analysis of low molecular weight compounds containing a variety of functional groups but was later applied to fatty acid methyl esters (14). From the data in Table III, it is evident that either of these corrections will greatly improve the accuracy of results obtained by isothermal operation. However, when these corrections are applied to the values obtained by temperature programming, there is a significant increase in the deviation, resulting in an analysis that is considerably less accurate.

These results would appear to show that under proper system conditions (temperature programming), the TC detector was measuring a value equal to weight percentage. However, the saturated mixture (NIH-"F") was prepared primarily as a standard for isothermal operation and in order to have nearly equal peak heights on the chromatogram for each component, the amount of the first component (shortest retention) was small and the amount of each component increased until the last component (longest retention) was more than 45% of the sample. In temperature-programmed analysis of this sample, the early components of the mixture have small peak heights in order to keep the peaks of the later components on the chromatogram because all peak widths are nearly equal by this procedure. This extreme in peak heights and individual component size could allow a relatively large error in measuring the area of the components with the shortest retention times (small peaks). These errors may give rise to an analysis such that it agrees well with the known weight percentage. A mixture of esters in which each was present in equal amounts would be expected to give a more accurate determination to show differences, if they exist, in cell response to various esters while temperature programming, although such a mixture is difficult to analyze isothermally.

The results of analyses of an equal weight mixture of methyl esters is given in Table IV. With this

TABLE II

Quantitative Reference Mixture—NIH "F"
Silicone Rubber—SE-30 Column

Ester	Known[c] wt. %	Isothermal[a]			Programmed temperature[b]		
		Found area %[d]	From known		Found area %[d]	From known	
			Dev.	% Dev.		Dev.	% Dev.
14:0	2.5	2.9 ± 0.1	+0.4	+16.0	2.6 ± 0.1	+0.1	+4.0
16:0	4.2	4.7 ± 0.1	+0.5	+11.9	4.3 ± 0.2	+0.1	+2.4
18:0	7.3	8.1 ± 0.3	+0.8	+10.9	7.5 ± 0.2	+0.2	+2.7
20:0	13.6	14.0 ± 0.2	+0.4	+ 2.9	13.7 ± 0.1	+0.1	+0.7
22:0	25.4	25.6 ± 0.3	+0.2	+ 0.8	25.5 + 0.2	+0.1	+0.4
24:0	46.6	44.7 ± 0.8	−1.9	− 4.1	46.4 ± 0.3	−0.2	−0.4

[a] Temp 225C; He flow 50 ml/min at exit; detector temp 256C.
[b] Temp 175C to 280C at 5.6C/min; He flow 50 ml/min at exit; detector temp 256C.
[c] Known also contained 0.4% C_{26}.
[d] Average of triplicate analysis.

TABLE III

Application of "Response Factors" for TC Cells
Reference Mixture NIH "F"—SE-30 Column

Ester	Known wt. %	Isothermal—225C			Prog. temp. 175–280C		
		Dev. from known			Dev. from known		
		Uncor.	Cor. A	Cor. B	Uncor.	Cor. A	Cor. B
14:0	2.5	+0.4	−0.2	−0.1	+0.1	−0.5	−0.4
16:0	4.2	+0.5	−0.1	0.0	+0.1	−0.5	−0.4
18:0	7.3	+0.8	+0.1	+0.2	+0.2	−0.4	−0.4
20:0	13.6	+0.4	−0.1	−0.1	+0.1	−0.5	−0.4
22:0	25.4	+0.2	+0.4	+0.3	+0.1	+0.3	+0.2
24:0	46.6	−1.9	+0.4	+0.1	−0.2	+2.0	+1.8

Correction A: $R = (24.68 + 5.79\ N - 0.075\ N^2)\ 1.019$

Correction B: $\left(\frac{m_i}{W}\right) = \frac{A_i\sqrt{M_i}}{\sum_i A_i\sqrt{M_i}}$

mixture, no significant difference in analyses was found when the column was held isothermally at 200C or when temperature programming was employed. In both cases, the analyses showed the usual trend toward high values for the lower molecular weight compounds and low values for the higher molecular weight compounds. These differences in cell response were not nearly as great as indicated from the isothermal analysis of NIH mix "F." Neither correction A nor B brought the values into line with the true weight percentages. Actually the factors overcorrected to an amount such that the deviation for each component was reasonably close numerically but with opposite signs than the value obtained for the uncorrected analysis of NIH mix "F." Neither correction A nor to 178C, the corrected values began to approach the true weight percentage.

A long-standing inconsistency in the molar response theory is the response of the unsaturated C_{18} methyl esters when adequately resolved. Isothermal GLC operation results in a decrease in response with increased unsaturation and therefore an apparent decrease in response with decreasing molecular weight. This is contrary to the molar response theory which would predict the opposite effect. This anomaly has been attributed to the effect on molar volumes by the *cis* double bonds (15).

The analysis of an unsaturated sample of fatty acid methyl esters (quantitative reference mixture H-105, Applied Science Laboratories, Inc.) is given in Table V. With isothermal operation, the deviations from the known weight percentage ranged from +0.7 to −1.0 and followed the usual trend of loss of area with increasing unsaturation. However, when the same sample was temperature-programmed, the range of deviations was only +0.1 to −0.1 which again represents a significant improvement in accuracy. This sample (H-105) contained esters in nearly equal amounts, and differences in cell response between components, if existent, should have been apparent in temperature-programmed analysis. That they were not could be interpreted to mean that unsaturation has very little, if any, effect on cell response. The molecular weight spread in this mixture was very small which would make differences due to molecular weight negligible.

Temperature-programmed operation in GLC analyses of fatty acid methyl esters generally resulted in percentages close to weight percentage and application of the proposed correction factors for "cell response" will often result in a less accurate analysis. The factors applied to isothermal GLC analysis usually will improve the analysis but, again, not in every case, and the correction required will depend on system conditions and sample composition.

As mentioned earlier, changes in molecular weight affect the thermal conductivity of a compound. It follows that the TC cell response theoretically should give mole percentage. Barsky (1) has an excellent discussion of various aspects of thermal conductivity as applied to GLC. He shows that the equation $K_{1,2} = X_1K_1 + X_2K_2$, where the subscripts 1 and 2 refer to two components of the mixture, X denotes mole fraction and K = thermal conductivity coefficient of the pure gases, is a simple additive equation expressing a linear relationship between thermal conduction and the mole composition of a binary gas mixture. The equation represents a fairly good approximation for certain specific situations, for example, where differences in molecular mass, collision diameters, and intermolecular forces are small. Barsky (1) points out that as the difference in mass and collision diameters in the mixture becomes large, there is a negative deviation and $K_{1,2}$ becomes smaller than predicted by the equation. Opposed to this tendency is the fact that when components of the mixture exhibit different intermolecular forces, the thermal conductivity coefficient of the mixture is enhanced or $K_{1,2}$ will be larger than predicted.

In the GLC analysis of a mixture of fatty acids or their esters, the detector is measuring a series of binary mixtures of helium and a component which have a greater and greater difference in molecular mass and collision diameters as the analysis proceeds. This in turn causes an increasingly greater difference in thermal conduction between the helium reference

TABLE IV

Equal weight mixture—This laboratory
SE-30 Column

Ester	Known wt. %	Isothermal—178C[a]			Isothermal—200C[a]			Programmed temp. 175–235C		
		Deviations from known								
		Uncor.	Cor. A[a]	Cor. B[b]	Uncor.	Cor. A[a]	Cor. B[b]	Uncor.	Cor. A[a]	Cor. B[b]
12:0	25.0	+2.7	−0.3	+0.5	+1.0	−2.0	−1.1	+1.0	−2.0	−1.1
14:0	25.0	+1.3	+0.6	+0.8	+0.4	−0.4	−0.2	+0.3	−0.5	−0.3
16:0	25.0	−1.0	+0.2	−0.2	−0.1	+1.0	+0.7	−0.1	+1.0	+0.6
18:0	25.0	−3.0	−0.4	−1.1	−1.3	+1.4	+0.7	−1.1	+1.5	+0.9

[a] All other conditions as shown in Table II.
[b] Same as shown in Table III.

TABLE V

Quantitative Reference Mixture—H105
Applied Science Laboratories, Inc.
EGS Column

Ester	Known wt. %	Isothermal 200C		Prog. temp 175–235C	
		Found area %	Dev. known	Found area %	Dev. known
18:0	26.9	27.3	+0.4	26.9	0.0
18:1	24.6	25.3	+0.7	24.7	+0.1
18:2	28.6	28.5	−0.1	28.6	0.0
18:3	19.9	18.9	−1.0	19.8	−0.1

and helium plus sample in the TC cell. This should result in a greater cell response for higher molecular weight components than would be predicted by the equation. The observed values show that this is the case and that the area percentage is usually closer to weight percentage than it is to mole percentage. For example, if we consider only the components 12:0 and 18:0 in the mixture of fatty acid methyl esters, shown in Table IV under temperature-programmed operation, we find that they were prepared in a weight ratio of 1.0 to 1.0. The calculated molar ratio is 1.0 to 0.72 but the observed area value is an intermediate ratio of 1.0 to 0.91 or close to weight percentage. However, if the analysis is performed under conditions that are not optimum, the ratio will approach mole percentage. For example, the observed area ratio in Table IV for these same esters, chromatographed isothermally at 178C, is 1.0 to 0.79 or close to mole percentage.

This could explain why there has been so much discussion in the literature whether the GLC results are weight or mole percentages or neither (2,3,8,13,16,17, 19,20,23,24). This also could explain why the proposed correction factors do not always apply. Since the basic detection system should give mole percentage but is unfortunately subject to deviations, it would seem logical that a correction factor should really be applied to bring the analysis to mole percentage but it is more common to report analyses in per cent by weight. The exact effect of increasing molecular weight or differing intermolecular forces on thermal conductivity is difficult to determine. Therefore, exact correction factors other than those determined empirically are unknown. These factors may be determined as described in the AOCS Tentative Method CE 1-62, revised, July 27, 1965 (22).

In addition to the above explanation, there are at least two other possible causes for the difference in analysis by isothermal and programmed-temperature operation as shown by the analysis of NIH mix "F" (Table II) and the equal weight mixture at the two temperatures, 178C and 200C (Table IV). The first would consider that every detector has a threshold sensitivity below which no signal is emitted and that every recorder and integrator requires a minimum signal to operate. This condition will cause a loss of area at the base of each peak. During isothermal runs, the peaks become progressively wider as the retention time increases and the loss increases proportionately. When the temperature is programmed so that all the peaks approach the same width, the losses will tend to be equalized. This is shown in Fig. 1. The area at the base of each peak enclosed by the dashed line represents the undetected portion of the sample. In the figure, these areas do not represent any known amount of loss but were made large merely to illustrate how this loss can occur. We have not evaluated this loss quantitatively and can only say that it exists in all detector-recorder-integrator systems and that the significance of this loss will depend on the total area of the peak. The second possible area loss, and likely the more significant especially when conditions are not optimum, would be the loss due to delay of components in the column packing as a result of solubility or other affinity for the stationary phase. Generally, component vapors having long retention times also have higher boiling points and tend to be more soluble in the stationary phase at a given temperature than those having shorter retention times (11). Possible loss in peak area as a result of solubility would be minimized by programming the temperature, thereby increasing the vapor pressure of the higher boiling components and thus reducing their solubility at the higher temperatures of operation.

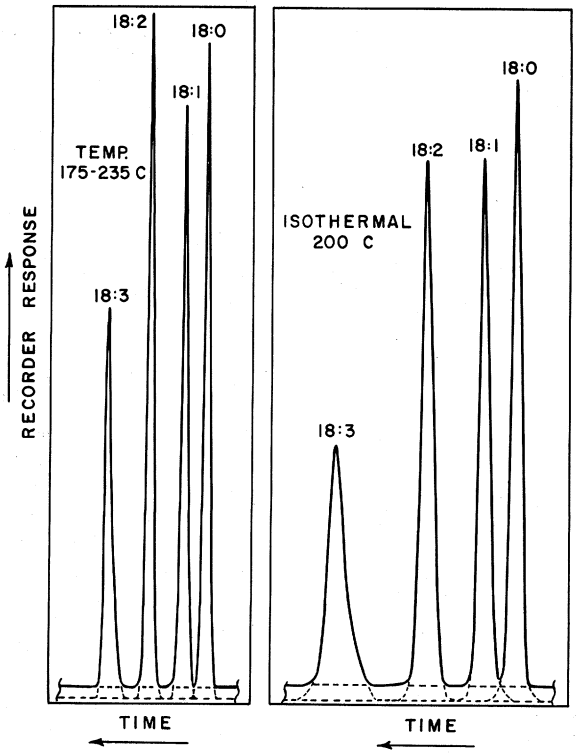

FIG. 1. Relative area loss due to sensitivity threshold.

The results of this study have indicated the complexity of the GLC system; that the behavior of one part of the system cannot be considered independently from the whole system and that proposed correction factors based on mass response of a thermal conductivity cell as being solely a simple function of molecular weight often lead to greater error than no corrections. The accuracy of analysis, either by isothermal or temperature-programmed GLC, may be improved by employing optimum system conditions. Precise analysis requires application of empirical correction factors. These factors should be determined under the same operating conditions used in the analysis and preferably with a known mixture of compounds which approximate the percentage found in the sample to be analyzed.

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.ures obtained by different methods should be compared with some caution. Preliminary observations suggest that even small differences can be shown by the present method between the denaturation temperatures of tropocollagen molecules with varying cross-linking.

This method has several advantages in the study of the temperature-linked changes in collagen. The procedure is easy, simple and fast. In contrast to the classical methods, every component in a mixture is analysed separately without any preceding fractionation process. The band pattern is conspicuous and its continuity through the temperature range renders it easy to assign the bands in spite of slight differences in the mobility. Because all the different temperatures are comprised on the same gel sheet, the occurrence of misleading artefacts is decreased.

The main drawbacks of the method are that it is less accurate than the classical methods and that the times and concentrations cannot be so well controlled. The presence of starch in the medium may affect the properties of the materials being studied. The choice of the ionic environment is also restricted[2].

The temperature gradient block can be also used for other purposes. The combination of a temperature gradient with the different "thin layer"-separation methods might be rewarding; for example, when the optimal temperature for a given separation is sought or when temperature-dependent processes are studied by "thin layer"-methods.

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SUMMARY

An apparatus is described for creating a temperature gradient for the combination with starch-gel electrophoresis or with "thin-layer" type separation methods.

The device is applied to studies on the reconstitution and thermal denaturation of collagen.

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